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NFkB is a transcription factor that functions to block the apoptotic response. Inappropriate activation of NFkB is thought to block apoptosis in breast cancer cells. NfkB activity is negatively regulated by a signaling pathway that responds to extracellular signals, including cytokines. Normally, NFkB is held in t cytoplasm by its inhibitor, IkB.In response to extracellular signals, IkB is destroyed by the process of ubiquitin mediated proteolysis. This process is activated through protein kinases that respond to cytokine such as TNFalpha. These kinases phosphorylate IkB, thereby activating it for ubiquitinaton. Ubiquitination involves 3 activities: an E1 activating enzyme, an E2 ubiquitin conjugating enzyme, and an E3 ubiquitin-protein ligase. In work supported by this grant, we have identified the molecular components involved in t ubiquitination. The ubiquitin ligase is composed of Skp1/Cul1/Rbx1 and the specificity factor beta-TRCP. Beta-TRCP binds IkB in a phosphorylation dependent manner and targets it for ubiquitnation via the SCF pathway. These data suggest that molecules which interfere with IkB ubiqutination by the SCFbeta-TRCP complex could function as anti-apoptotic agents.

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FOREWORD

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Jeffrey Harper, Ph.D. 6/7/00

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Introduction

The process of apoptosis is critical to the development and homeostasis of multicellular organisms. It provides a mechanism for loss of cells during organogenesis and provides a pathway for removal of cells that are undergoing inappropriate proliferative events or have receive unrepairable DNA damage. A theme in cancer biology is that cellular transformation requires the establishment of survival pathways that limit the process of apoptosis. Recent studies have revealed that one such survival pathway is established through the action of the transcription factor NFkB. NFkB plays important roles in activation of genes in response to cytokines and other stimuli, and has been well characterized with respect to its role in inflammatory diseases. Cytokines such as TNF-α activate two pathways, one that activates a cell death response, and one that activates a survival response that is dependent upon NFkB. This finding has renewed interest in the development of inhibitors of the NFkB pathway that can be used therapeutically to block survival pathways while simultaneously allowing for agents such as TNF or chemotherapeutics to activate apoptotic pathways. The use of such combination therapy has the potential to allow proliferative control of established tumors. Because of the widespread nature of the NFkB survival pathway, such NFkB inhibitors would be expected to be useful in a wide variety of proliferative diseases and mammary cancer is no exception. Recent studies have revealed that mammary tumor cells utilize NFkB in an anti-apoptotic mechanism and display increased NFkB activity that correlates with estrogen-independence. NFkB function is normally controlled by IkB, which holds NFkB in the cytoplasmic. Signals which activate NFkB lead to phosphorylation of IkB, which signals it for ubiquitin-mediated proteolysis thereby allowing NFkB to enter the nucleus. The importance of IkB in the survival function of NFkB is demonstrated by the fact that overexpression of non-phosphorylatable IkB blocks NFkB function, allowing for apoptosis.

Although it is known that phosphorylation of IkB is required for its ubiquitination, the machinery involved is unknown. However, because of its critical position in the NFB activation pathway, the IkB ubiquitin ligase represents an important therapeutic target. Blocking its activity would be equivalent to overexpression of non-phosphorylatable IkB and would be expected to lead to inhibition of NFkB. In work funded by this grant, we have identified a protein complex, $SCF^{\beta\text{-}TRCP}$, which we have shown to function in the targeted ubiquitination of IkB. SCF complexes function as E3 ubiquitin ligases and are composed of Skp1, the Ring-H2 finger protein Rbx1, the Cul1 protein, and an F-box protein, in this case β-TRCP. This complex recognized IkB in a phosphorylation-dependent manner and catalyzes IkB ubiquitination in vitro, in conjunction with an E2 ubiqutin conjugating enzyme and an E1 activating enzyme. This was a major goal of aim 1 of this proposal and now sets the stage for further studies aimed at understanding how $SCF^{\beta\text{-}TRCP}$ functions in IkB ubiquitnation and whether this complex serves as a point of thereapeutic intervention with the aim of blocking anti-apoptotic pathways in tumor cells.

Body

Because the major results of our studies have recently been published, I will simply summarize the results. A copy of the papers are provided in the appendix. Using biochemical and molecular biological approaches, we established the following facts:

1. Peptides containing the IkB destruction motif can interact with a complex containing Skp1 in vitro and this interaction requires that the IkB motif known to be involved in phosphorylation-dependent ubiqutination be phosphorylated on both Ser-32 and serine-36. (see Figure 1 in Genes Dev. 13, 270, appendix)

2. The F-box protein β-TRCP selectively interacts with phosphorylated IkB destruction motifs. (see Figure 1 in Genes Dev. 13, 270, appendix)

3. β-TRCP is expressed throughout the developing embryo and is located in the cytoplasm, as expected for the molecule responsible for IkB destruction. b-TRCP maps to 10q24 in the human genome. (see Figure 1 in Genes Dev. 13, 270, appendix)

4. β-TRCP associates with SCF complexes. Using transfection, we demonstrated that β-TRCP will assemble into SCF complexes and that these complexes will associate with both the IkB destruction motif and with assembled IkB/NFkB compelxes in a phosphorylation dependent manner. (see Figure 2-3 in Genes Dev. 13, 270, appendix)

5. $SCF^{\beta\text{-}TRCP}$ co-fractionates with IkB ubiquitin ligase activity purified from HeLa cells. (see Figure 4 in Genes Dev. 13, 270, appendix)

6. Depletion of $SCF^{\beta\text{-}TRCP}$ correlates with the loss of IkB ubiquitin ligase activity from HeLa cell extracts. (see Figure 5 in Genes Dev. 13, 270, appendix)

7. β-TRCP stimulates the ubiquitnation of IkB in vitro in a phosphorylation dependent manner. (see Figure 6 in Genes Dev. 13, 270, appendix)

8. A common F-box protein, β-TRCP, function in both the IkB and β-catenin destruction pathways. β-catanin is a gene frequently found mutated in colon cancer and its acumulation activates the expression on oncogenes.

9. Two closely related β-TRCP homologs exist in the human genome. (see Figure 1 in Curr. Biol.9, 1180)

Discussion

Activation of NFkB involves an extensive signal transduction pathway that culminates in the destruction of the NFkB inhibitor IkB. Although the protein kinase pathways that control the timing of NFkB activation have been defined, the molecules

responsible for the actual ubiquitination events have not been elucidated. Inappropriate NFkB activation frequently occurs in various types of cancers, including beasr cancers, and functions in this context to block apoptosis. In this work, we provide biochemical evidence that the WD-40-containing F-box protein, β-TRCP, functions as a specificity factor in an SCF complex to promote signal dependent ubiquitination of IkB (Figure 7 in Genes and Dev. 13, 270 in appendix). A role for β-TRCP in controlling IkB ubiquitination is supported by the following findings. (1) Destruction of IkB is known to require IKK-dependent phosphorylation of residues (Ser32 and Ser36) located in a destruction motif. β-TRCP and its SCF complex associate with this IkB destruction motif and with the IkB/NFkB complex in a manner that is dependent upon phosphorylation of the IkB destruction motif. A variety of other F-box proteins, including two other WD-40 containing F-box proteins (Met30 and MD6), failed to associate with either phosphorylated or unphosphorylated IkB destruction motifs, pointing to the specificity of the interaction with β-TRCP. We believe that the interaction between β-TRCP and the IkB destruction motif is direct since peptide beads containing this motif precipitate GST-β-TRCP from insect cell lysates in the absence of other abundant proteins (unpublished data). (2) β-TRCP forms a complex with two proteins, Skp1 and Cul1, that have previously been linked to phosphorylation dependent ubiquitination. (3) β-TRCP co-purifies with IkB ubiquitin-ligase activity from tissue culture cells and these active fractions also contain Cul1 and Skp1. (4) Depletion of β-TRCP with either anti-Skp1 antibodies or phosphorylated destruction motif peptides correlates with loss of IkB ubiquitin-ligase activity. (5) SCF$^{\beta\text{-TRCP}}$ complexes stimulated phosphorylation dependent IkB ubiquitin ligase activity when supplemented with E1, ubiquitin, ATP, and a yeast extract. These yeast extracts lack IkB ubiquitin ligase despite the presence of multiple SCF complexes (Bai et al., 1996; Patton et al., 1998a,b), providing further evidence of a role for β-TRCP as a specificity factor for IkB, but provide E2 activities and possibly other components that support IkB ubiquitination by the β-TRCP complex. At present, we have been unable to reconstitute IkB ubiquitin ligase activity using SCF$^{\beta\text{-TRCP}}$ complexes isolated from transfected cells and column fractions depleted of β-TRCP by either anti-Skp1 antibodies or phospho-IkB peptides. This may reflect removal of an essential factor by depletion which is not present in sufficient levels in the transiently expressed SCF complex to support IkB ubiquitination, but which are provided in trans by yeast extracts. Taken together, our data strongly indicate that IkB ubiquitination is mediated by the SCF$^{\beta\text{-TRCP}}$ complex where β-TRCP functions in IkB recognition. Currently, the identity of the E2(s) involved in IkB ubiquitination in vivo is unknown, as is the nature of the heterogeneity observed with β-TRCP. We note, however, that other F-box proteins including Skp2 are modified by phosphorylation (Lisztwan et al., 1998) and such modifications could potentially play regulatory roles. The methods we have employed offer two general approaches to determining whether a particular ubiquitination process involves an SCF complex: (1) depletion of active fractions with Skp1 antibodies and (2) the use of substrates as affinity reagents to examine association with cloned F-box proteins.

The sequence conservation of the IkB destruction motif with a region of β-catenin implicated in its turnover, coupled with a genetic requirement for the β-TRCP homolog slimb in turnover of the β-catenin homolog armadillo (Jiang and Struhl,

1998), led us to address whether β-TRCP might interact directly with β-catenin. Phosphorylation of serine residues 33 and 37 was sufficient to allow for a peptide spanning this region to associate with β-TRCP and its SCF complex, but not other F-box proteins. β-catenin is a component of the Wingless/Wnt signaling pathway and functions with Tcf/Lef transcription factors to regulate patterning and other developmental decisions. Recent work in *Xenopus* has revealed that expression a β-TRCP protein lacking the F-box leads to accumulation of β-catenin and ectopic activation of the Wnt pathway (Marikawa and Elinson, 1998). This, together with our data linking β-TRCP to direct recognition of the phosphorylated β-catenin destruction motif strongly implicates $SCF\beta^{-TRCP}$ as the β-catenin ubiquitin ligase (Figure 7). The levels of β-catenin are regulated by the APC (adenomatous polyposis coli) tumor suppressor protein, axin, and the protein kinase GSK3β (Korinek et al., 1997; Morin et al., 1997; Rubinfeld et al., 1997). Formation of an APC/axin/GSK3β/β-catenin complex is thought to be required to allow appropriate phosphorylation of β-catenin by GSK3β (Hart et al., 1998; Ikeda et al., 1998) and in the absence of Wnt signaling, β-catenin levels remain low due to constitutive phosphorylation and ubiquitin-mediated proteolysis. Wnt signaling inactivates GSK3β, leading to increased levels of β-catenin and activation of transcription. Mutations in either the APC gene or in β-catenin allow for β-catenin accumulation (Morin et al., 1997; Rubinfeld et al., 1997). Such mutations are found in a large fraction of colon cancers (Morin et al., 1997) and have also been observed in melanoma (Rubinfeld et al., 1997), prostate cancer (Voeller et al., 1998), and experimentally induced cancers (Dashwood et al., 1998). Interestingly, stabilizing mutations in β-catenin are localized to its destruction motif and include mutations in both the phosphoacceptor sites S33 and S37 and other residues in the consensus β-TRCP recognition motif including D32, G34, and I35. Mutations in these residues would be expected to weaken or abolish association of β-catenin with β-TRCP, leading to increased β-catenin levels.

The role for β-TRCP in β-catenin turnover suggests that it might function as a tumor suppressor. We localized the human β-TRCP gene to 10q24. This region displays genetic abnormalities in a limited number of prostatic, melanocytic, and neural cancers (Lundgren et al., 1992; Parmiter et al., 1988; Rasheed et al., 1992). However, a preliminary analysis of 4 colon cancers which are wild-type for β-catenin and APC failed to reveal mutations in the β-TRCP protein (Sparks et al., 1998). Given the role for β-TRCP in IkB ubiquitination, it is conceivable that mutations that inactivate β-TRCP are incompatible with transformation because of loss of survival pathways dependent upon NFkB activation (Beg et al., 1996; Van Antwerp et al., 1996; Wang et al., 1996).

Our results indicate that an $SCF\beta^{-TRCP}$ complex functions in two critical transcriptional control pathways, in one case by inactivating an inhibitor of transcription and in the other case by inhibiting an activator of transcription ((Figure 7 in Genes and Dev. 13, 270 in appendix). Genetic data in Drosophila suggests that the β-TRCP homolog slimb may also regulate the hedgehog pathway (Jiang and Struhl, 1998). In *slimb* mutants, the Ci transcription factor accumulates inappropriately, although the question of whether this regulation is direct remains to be determined. Moreover, other recent studies have revealed that β-TRCP is co-opted by the HIV

protein vpu to facilitate destruction of the CD4 protein (Margottin et al., 1998). Interestingly, vpu contains a β-TRCP recognition motif very similar to that found in IkB and β-catenin and phosphorylation is required for it to recruit CD4 to β-TRCP and to bind β-TRCP in the two-hybrid system. Further studies are required to determine whether any of the many proteins containing the DSGϕXS motif are also substrates for $SCF^{\beta\text{-}TRCP}$. In addition, we note that the anti-inflammatory affects of aspirin are mediated through inhibition of IkB activity (Grilli et al., 1996; Yin et al., 1998), thereby blocking NFkB activation. Molecules that selectively block recognition of IkB by β-TRCP may also constitute an alternative therapeutic target for anti-inflammatory agents.

Research Accomplishments:

* Identification of the IkB ubiquitin ligase
* Demonstration that the $SCF^{\beta\text{-}TRCP}$ complexes recongizes IkB in a phosphorlation dependent manner
* Identification of the β-catenin ubiquitin ligase
* Identification of a second β-TRCP gene in the human genome

Reportable outcomes.

Publications:

Winston, J.T., Strack, P., Beer-Romero, P., Chu, C., Elledge, S.J., and Harper, J.W. (1999) The$SCF^{\beta\text{-}TRCP}$ ubiquitin ligase specifically associates with phosphorylated destruction motifs in IkB and β-catenin and stimulates IkB ubiquitination in vitro. **Genes and Development**, 13, 270-283. (recognized as a "Hot Paper" by ISI, ranked 5th among all papers for citations in 1999)

Accompanying minireviews in **Genes and Development** and in **Cell**
" Tom Maniatis (1999) A ubiquitin ligase complex essential for the NF-kB, Wnt/Wingless, and Hedgehog signaling pathways. **Genes & Dev**. 13: 505-510."
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Winston, J.T., Koepp, D.M., Zhu, C., Elledge, S.J., and Harper, J.W. (1999) A family of mammalian F-box proteins. **Current Biology** 9, 1180-1182.

Conclusion

Ongoing and future studies.

This report describes our efforts on aim 1 of the proposal. Aim 2 seeks to understand how β-TRCP interacts with IkB, with the hope of exploiting this interaction to block NFkB activation. In principle, small moleculaes that block the association of IkB with β -TRCP could block the anti-apoptotic activities of NFkB by maintaining it in the inactive form bound to IkB. In preliminary experiments, we have modeled the structure of β -TRCP onto the structure of another WD40 repeat protein (β -transducin) and we are currently using this model to design mutants that may affect the interaction of β -TRCP with IkB. These studies will be described in greater detail in next year's report.

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The $SCF^{\beta\text{-}TRCP}$–ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IκBα and β-catenin and stimulates IκBα ubiquitination in vitro

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Ubiquitin-mediated proteolysis has a central role in controlling the intracellular levels of several important regulatory molecules such as cyclins, CKIs, p53, and IκBα. Many diverse proinflammatory signals lead to the specific phosphorylation and subsequent ubiquitin-mediated destruction of the NF-κB inhibitor protein IκBα. Substrate specificity in ubiquitination reactions is, in large part, mediated by the specific association of the E3–ubiquitin ligases with their substrates. One class of E3 ligases is defined by the recently described SCF complexes, the archetype of which was first described in budding yeast and contains Skp1, Cdc53, and the F-box protein Cdc4. These complexes recognize their substrates through modular F-box proteins in a phosphorylation-dependent manner. Here we describe a biochemical dissection of a novel mammalian SCF complex, $SCF^{\beta\text{-}TRCP}$, that specifically recognizes a 19-amino-acid destruction motif in IκBα (residues 21–41) in a phosphorylation-dependent manner. This SCF complex also recognizes a conserved destruction motif in β-catenin, a protein with levels also regulated by phosphorylation-dependent ubiquitination. Endogenous IκBα–ubiquitin ligase activity cofractionates with $SCF^{\beta\text{-}TRCP}$. Furthermore, recombinant $SCF^{\beta\text{-}TRCP}$ assembled in mammalian cells contains phospho-IκBα-specific ubiquitin ligase activity. Our results suggest that an $SCF^{\beta\text{-}TRCP}$ complex functions in multiple transcriptional programs by activating the NF-κB pathway and inhibiting the β-catenin pathway.

[*Key Words*: Ubiquitin ligase; SCF complex; proteolysis; destruction motifs; NF-κB; β-catenin]

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The transcription factor NF-κB has a central role in cellular stress and inflammatory responses by controlling cytokine-inducible gene expression and lymphocyte stimulation by antigens (Baeuerle and Baltimore 1996; Gilmore et al. 1996). In addition, NF-κB is required to block cell death in response to tumor necrosis factor α (TNFα) and ionizing radiation, suggesting that it acts to regulate the transcription of survival genes (Beg and Baltimore 1996; Liu et al. 1996; Van Antwerp et al. 1996; Wang et al. 1996). NF-κB is a ubiquitous heterodimeric complex composed of a p65/RelA subunit and a p50 subunit. This complex is normally sequestered in an inactive form in the cytoplasm through interaction with members of a family of inhibitory proteins, the IκBs (Beg et al. 1992; for review, see Baeuerle and Baltimore 1996). These proteins, when associated with NF-κB, obscure the nuclear localization signal in NF-κB and also block the ability of NF-κB to bind DNA. In response to TNFα and other signals, IκBα is rapidly phosphorylated on two serine residues near the amino terminus (Ser-32 and Ser-36 in IκBα) (Beg et al. 1993; Finco et al. 1994; Alkalay et al. 1995; Brown et al. 1995; Chen et al. 1995, 1996; DiDonato et al. 1995; Lin et al. 1995). This modification triggers the rapid destruction of IκBα by ubiquitin-mediated proteolysis, thereby allowing NF-κB nuclear translocation and target gene expression (Chen et al. 1995; Scherer et al. 1995; for review, see Hochstrasser 1996). Recent work has uncovered two IκBα kinases, IκKα and IκKβ, that are responsible for signal-dependent phosphorylation of IκBα (DiDonato et al. 1997; Mercurio et al. 1997; Regnier et al. 1997; Woronicz et al. 1997; Zandi et al. 1997, 1998). These proteins are part of a 700-kD protein complex that is assembled through two structural components IκKγ/NEMO and IKAP (Cohen et al. 1998; Rothwarf et al. 1998; Yamaoka et al. 1998) and are activated by cytokines. In vitro, both IκKα and IκKβ can

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phosphorylate IκBα specifically on serines 32 and 36, but both kinases are required for efficient IκBα phosphorylation in vivo (Zandi et al. 1997).

Although the pathways leading to IκBα phosphorylation have been described in detail, little is known about the molecules responsible for ubiquitination. Ubiquitin-mediated proteolysis involves a cascade of ubiquitin transfer reactions in which the ubiquitin-activating enzyme E1 uses ATP to form a high-energy thiolester bond with ubiquitin, which is then transferred to members of the E2 ubiquitin-conjugating enzyme family (Hershko et al. 1983; Hochstrasser 1995). Ubiquitin is then transferred from the E2 to lysine residues in the target through an E3–ubiquitin ligase. E3s serve as adaptors that interact with both the target protein and the appropriate E2, thereby providing specificity to the ubiquitin transfer reaction. In some cases, the E3 is also involved in ubiquitin transfer (Scheffner et al. 1995; Rolfe et al. 1995). Multiple rounds of ubiquitination of the initial conjugates lead to polyubiquitination, which targets the protein for proteolysis by the 26S proteasome. Recent studies have elaborated a modular ubiquitin ligase complex, the SCF–ubiquitin ligase, which mediates phosphorylation-dependent ubiquitination of a large number of proteins (for review, see Elledge and Harper 1998; Patton et al. 1998b). The SCF is composed of Skp1, Cdc53/Cul1, and a specificity-conferring F-box protein (Bai et al. 1996; Feldman et al. 1997; Skowyra et al. 1997; Patton et al. 1998a). F-box proteins contain two domains, an F-box motif that binds Skp1 and allows assembly into Skp1/Cdc53 complexes, and a second protein–protein interaction domain that interacts specifically with one or more target proteins (Bai et al. 1996). Cdc53/Cul1, in turn, interacts with both the E2 and the Skp1/F-box protein complex (Skowyra et al. 1997; Patton et al. 1998a). SCF complexes mediate phosphorylation-dependent destruction of a wide array of regulatory proteins in yeast, including the Cdk inhibitors Sic1, Far1, and Rum1, G_1 cyclins, the transcription factor Gcn4, and the DNA replication initiator proteins Cdc6 and Cdc18 (for review, see Elledge and Harper 1998; Patton et al. 1998b). In contrast with yeast, targets of vertebrate SCF complexes remain largely unknown. Previously, we identified four vertebrate proteins that contain the F-box motif, linking them to the ubiquitin pathway: mammalian cyclin F, Skp2, MD6, and *Xenopus* β-TRCP (β-transducin repeat-containing protein; Bai et al. 1996). β-TRCP was originally identified as a suppressor of a temperature-sensitive mutation in the budding yeast *CDC15* gene (Spevak et al. 1993), but its mechanism of suppression has not been determined. Recent genetic evidence has implicated *Xenopus* β-TRCP and its *Drosphila* homolog, *slimb*, in control of proteolysis in the Hedgehog and Wingless/Wnt signaling pathways (Jiang and Struhl 1998; Marikawa and Elinson 1998).

We have used biochemical approaches to examine whether IκBα ubiquitination might involve an SCF–ubiquitin ligase. Here we report that mammalian β-TRCP binds to the IκBα destruction motif in a phosphorylation-dependent manner, thereby recruiting IκBα into an SCF–ubiquitin ligase complex. Moreover, $SCF^{\beta\text{-}TRCP}$ components cofractionate with IκBα–ubiquitin ligase activity from tissue culture cells and $SCF^{\beta\text{-}TRCP}$ can stimulate ubiquitination of phosphorylated but not unphosphorylated IκBα in an in vitro reconstitution assay. We also demonstrate that the same $SCF^{\beta\text{-}TRCP}$ complex recognizes a similar destruction motif in β-catenin, a component of the TCF/Lef transcription factor complex that functions downstream of Wingless/Wnt (for review, see Peifer 1997) and whose levels are also controlled by phosphorylation-dependent ubiquitin-mediated proteolysis (Aberle et al. 1997). Our results, together with the effects of loss-of-function mutations in the *Drosophila* β-TRCP homolog *slimb* (Jiang and Struhl 1998), suggest that a single $SCF^{\beta\text{-}TRCP}$ complex functions in diverse signaling pathways that impinge on transcription control mediated by cytokines (NF-κB), Wnt/Wingless (β-catenin), and Hedgehog [Cubitus interruptus (Ci)].

Results

Phosphorylation-dependent association of the IκBα destruction motif with Skp1

IκBα contains two serine residues at positions 32 and 36 that are specifically phosphorylated by the IκK complex in response to TNFα stimulation. Phosphorylation of both of these residues is required for IκBα ubiquitination in vivo. Previous studies have shown that a 21-amino-acid phosphopeptide containing this destruction motif can block IκBα–ubiquitin ligase activity in crude cell extracts and can block NF-κB activation in tissue culture cells (Yaron et al. 1997). In addition, this destruction motif can confer signal-dependent destruction when fused to heterologous proteins (Wulczyn et al. 1998). Given the role for SCF complexes in phosphorylation-dependent ubiquitination of various regulatory proteins, we sought to determine whether SCF complexes might be involved in IκBα ubiquitination. Synthetic 21-residue peptides encompassing the IκBα destruction motif in either the doubly phosphorylated or unphosphorylated forms (Fig. 1a) were immobilized on agarose beads and incubated with HeLa cell lysates. Proteins stably associated with these beads were then examined for the presence of Skp1 by immunoblotting (Fig. 1b). Skp1 was readily detected in proteins bound to the phospho-IκBα peptide but not the unphosphorylated peptide. We estimate that ~1% of the total Skp1 in these lysates stably associated with the phospho-IκBα peptide under these conditions.

Recognition of phosphorylated destruction motifs in IκBα and β-catenin by the WD-40 repeat-containing F-box protein β-TRCP

The ability of a phospho-IκBα peptide to associate with Skp1 suggested the existence of an F-box protein capable of recognizing the IκBα destruction motif. Our previous studies identified three vertebrate F-box proteins (Skp2,

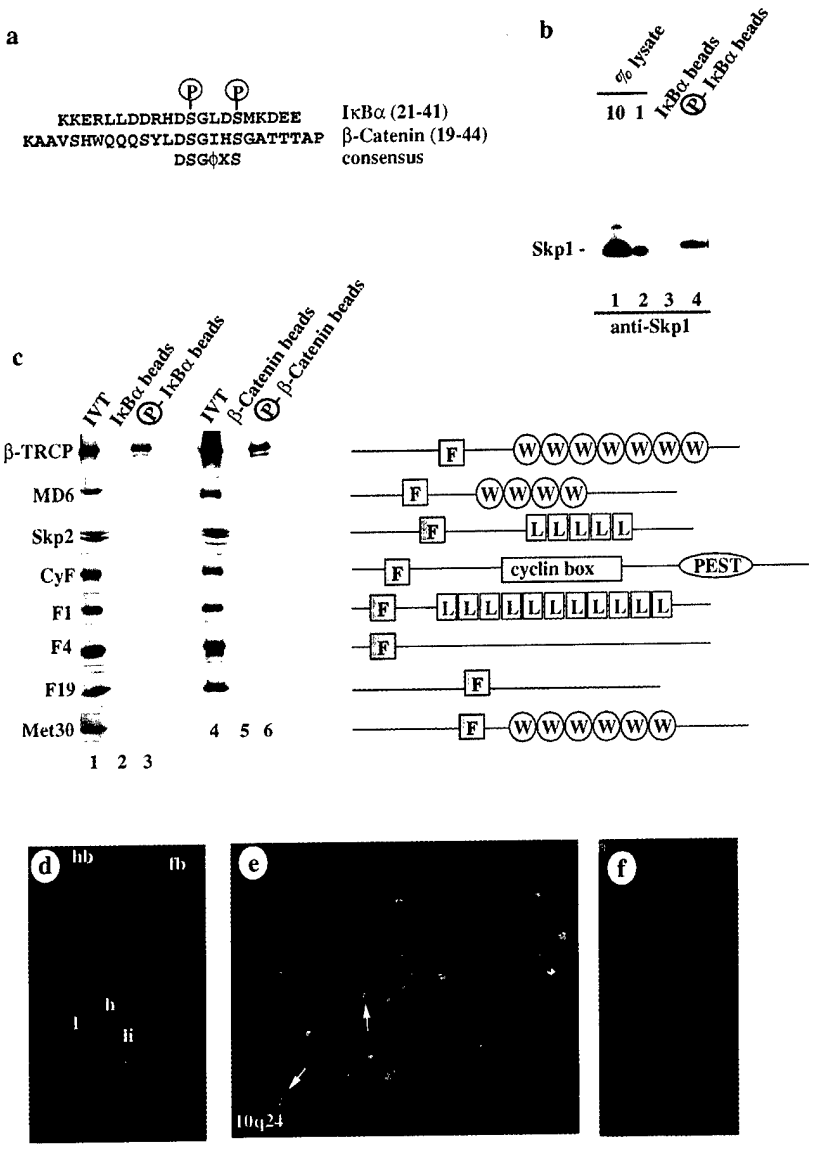

Figure 1. The F-box protein β-TRCP associates with phosphorylated destruction motifs in IκBα and β-catenin. (*a*) Sequences of the IκBα (p21) and β-catenin peptides used in this study. The positions of phosphorylation in the peptides are shown as is the consensus sequence for the destruction motif. (ϕ) Hydrophobic amino acid. (*b*) Phosphorylation-specific association of the IκBα destruction motif with Skp1 in vitro. HeLa cell proteins (600 μg) were incubated with phosphorylated or unphosphorylated IκBα peptides (p21) immobilized on beads. Bound proteins were immunoblotted with anti-Skp1 antibodies. Approximately 1% of the Skp1 contained in these lysates remained bound to the phosphorylated IκBα beads. (*c*) β-TRCP specifically associated with phosphorylated IκBα and β-catenin destruction motifs. A panel of [^{35}S]methionine-labeled in vitro-translated F-box proteins was used in binding reactions with IκBα (lanes *1–3*) or β-catenin (lanes *4–6*) peptide beads. Bound proteins were analyzed by SDS-PAGE and autoradiography. (*Right*) The domain structures of each F-box protein. (*d*) The pattern of expression of β-TRCP at day 11.5 during mouse development was determined by in situ hybridization. The dark-field signal from the β-TRCP riboprobe is shown in red. (hb) Hindbrain; (fb) forebrain; (h) heart; (l) lung; (li) liver. (*e*) Chromosomal localization of β-TRCP. A bacmid containing human β-TRCP DNA was hybridized to metaphase chromosomes (blue) and detected using fluorescein. The position of hybridization (yellow) is 10q24 (indicated by arrows). (*f*) β-TRCP is localized in the cytoplasm. HeLa cells were transiently transfected with pCMV–HA–β-TRCP and subcellular localization determined after 48 hr by indirect immunofluorescence. Anti-HA localization, red; nuclei stained with DAPI, blue.

MD6, and *Xenopus* β-TCRP) based on homology to the F-box sequence in human cyclin F and the budding yeast protein Cdc4 (Bai et al. 1996). Recently, we have identified cDNAs encoding 20 distinct mouse and/or human F-box proteins, including the WD-40 repeat-containing protein β-TRCP, a leucine-rich repeat containing F-box protein F1, and a number of F-box proteins lacking known protein–protein interaction motifs outside the F-box (Fig. 1c; J. Winston, S.J. Elledge, and J.W. Harper, in prep.). Using in vitro translation products, we asked whether members of a collection of these F-box proteins could associate with IκBα peptides. Only one, β-TRCP, was found to associate with the phospho-IκBα destruction motif, and this interaction was dependent on phosphorylation (Fig. 1c). Mouse and human β-TRCP are 95% identical and both interact equally well with IκBα in this assay (data not shown for human β-TRCP). Our analysis included two other WD-40-containing F-box proteins, human MD6 and Met30, the closest homolog of β-TRCP in budding yeast (31% identity). Importantly, neither of these proteins associated with phospho-IκBα (Fig. 1c), suggesting that the interaction of β-TRCP with phospho-IκBα is highly specific.

Previous studies in *Drosophila* have demonstrated that mutations in the β-TRCP homolog *slimb* led to accumulation of Armadillo, the *Drosophila* homolog of β-catenin (Jiang and Struhl 1998). β-Catenin is known to be ubiquitinated in a glycogen synthase kinase 3β (GSK3β)-dependent manner and contains a motif within a cluster of candidate GSK3β phosphorylation sites that is closely related to the IκBα destruction motif (Fig. 1a; Ikeda et al. 1998). Although the GSK3β phosphorylation sites in β-catenin are not known, we hypothesized based on the sequence similarity between IκBα and β-catenin that Ser-33 and Ser-37 might represent relevant phosphorylation sites. A β-catenin-derived peptide containing phosphoserine residues at these two positions associated with β-TRCP but not other F-box proteins tested,

whereas the unphosphorylated peptide failed to associate with β-TRCP (Fig. 1c).

NF-κB is a ubiquitous transcription factor. As assessed by in situ hybridization, β-TRCP is also expressed throughout the developing mouse embryo (day 11.5 postcoitum), with the highest levels in the brain, lung, and liver (Fig. 1d). β-TRCP is largely, if not exclusively, cytoplasmic, as assessed in HeLa cells transiently expressing an HA-tagged β-TRCP protein (Fig. 1f). The gene for human β-TRCP lies on chromosome 10q24, as determined by in situ hybridization of metaphase chromosomes with β-TRCP genomic DNA (Fig. 1e). Cytogenetic data indicate that this region of the genome is altered in a limited number of cancer types (see Discussion).

Association of SCF$^{\beta\text{-TRCP}}$ with IκBα and β-catenin destruction motifs

Having identified β-TRCP as a candidate F-box protein for IκBα and β-catenin, we next sought to demonstrate that β-TRCP forms an SCF complex in mammalian cells and that this complex recognizes IκBα and β-catenin destruction motifs. Although there are six mammalian Cullin homologs, the interaction of Skp1 with this family appears to be restricted to Cul1 (Michel and Xiong 1998). 293T cells were transfected with various combinations of plasmids expressing epitope-tagged β-TRCP, Cul1, or Skp1 and anti-Myc immune complexes from cell lysates analyzed by immunoblotting. β-TRCP^{Myc} associated with both transfection-derived Skp1^{HA} (Fig. 2a, lanes 4,5) and Cul1^{HA} (lane 5). In contrast, Cul1^{HA} and Skp1^{HA} were not precipitated from control lysates lacking β-TRCP^{Myc} (lane 6). In the absence of transfection of Skp1 and Cul1, β-TRCP^{Myc} associated with endogenous Skp1 (lane 3) and Cul1 (data not shown). Analogous results were obtained when Cul1^{HA} was immunoprecipitated with anti-HA antibodies from cells expressing Cul1^{HA}, β-TRCP^{Myc}, and Skp1^{Myc} (Fig. 2c). Thus, β-TRCP can form an SCF complex in vivo analogous to that found previously for other F-box proteins (Skowyra et al. 1997; Lisztwan et al. 1998; Lyapina et al. 1998; Michel and Xiong 1998).

Next, we asked whether the SCF$^{\beta\text{-TRCP}}$ complex could associate with the IκBα destruction motif peptide. As shown in Figure 3a, the SCF$^{\beta\text{-TRCP}}$ complex readily associated with phosphorylated IκBα peptide beads (lanes 6,8,10) but was not retained on unphosphorylated IκBα beads (lanes 5,7,9). Although Cul1 associates at low levels with agarose beads containing IκBα peptides in the absence of β-TRCP^{Myc} expression (lanes 11,12) and with agarose beads alone (data not shown), the association with the phospho-IκBα peptide was greatly enhanced by expression of β-TRCP^{Myc} (lane 10). Consistent with the results in Figure 1b, endogenous Skp1 was observed in

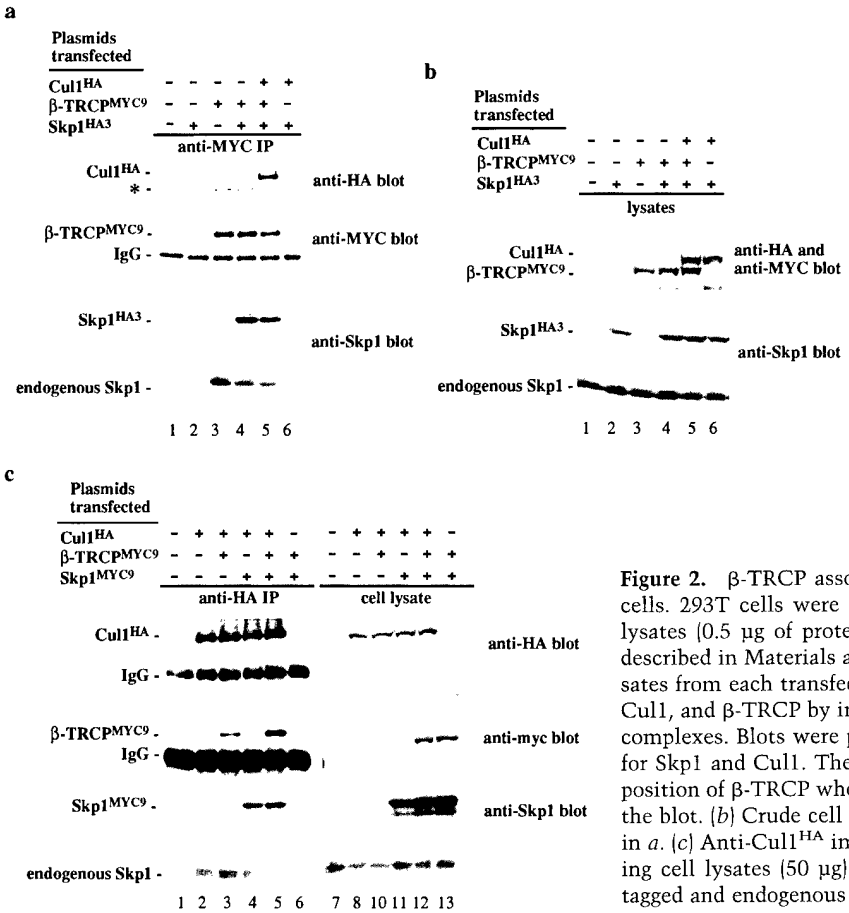

Figure 2. β-TRCP associates with Skp1 and Cul1 in tissue culture cells. 293T cells were transfected with the indicated plasmids and lysates (0.5 μg of protein/250 μl) used for immunoprecipitation as described in Materials and Methods. Immune complexes or crude lysates from each transfection were analyzed for the presence of Skp1, Cul1, and β-TRCP by immunoblotting. (*a*) Anti-β-TRCP^{Myc} immune complexes. Blots were probed first for β-TRCP, stripped, and probed for Skp1 and Cul1. The bands indicated by the asterisk indicate the position of β-TRCP whose antibody was not efficiently stripped from the blot. (*b*) Crude cell lysates (50 μg) corresponding to extracts used in *a*. (*c*) Anti-Cul1^{HA} immune complexes (lanes *1–6*) and corresponding cell lysates (50 μg) (lanes *7–13*). The positions of both epitope-tagged and endogenous Skp1 are shown.

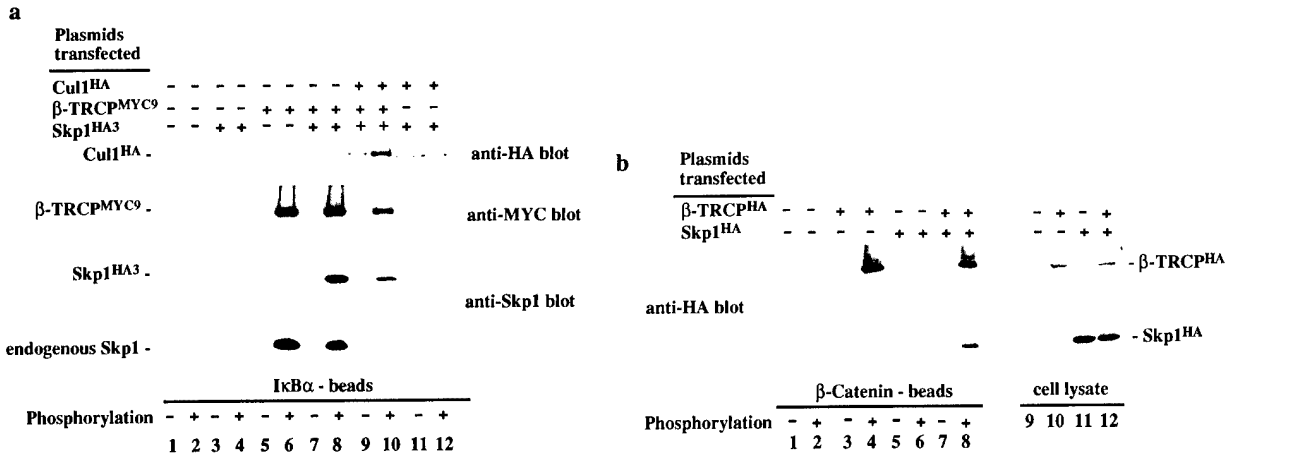

Figure 3. Association of $SCF^{\beta\text{-}TRCP}$ with IκBα and β-catenin destruction motifs and with the IκBα/NF-κB complex. (*a*,*b*) Cell lysates (0.3 μg of protein/150 μl) from Fig. 2 were used in IκBα (*a*) and β-catenin (*b*) peptide bead binding reactions as described in Materials and Methods. Bound proteins were analyzed by immunoblotting with the indicated antibodies. (*c*) Phosphorylation-dependent association of $\beta\text{-TRCP}^{Myc}$ with the IκBα/p50/p65 complex in vitro. $\beta\text{-TRCP}^{Myc}$ immune complexes (lanes *2*,*5*) corresponding to those in Fig. 2a (lane *3*) or control complexes (lanes *3*,*6*) corresponding to those in Fig. 2a (lane *1*) were used in binding reactions with either IκBα/p50/p65 or IκK-β phosphorylated IκBα/p50/p65 complexes (see Materials and Methods). Bound proteins were separated by SDS-PAGE and immunoblotted using anti-p50 or anti-IκBα antibodies. The asterisk (lanes *1*,*4*) indicates the positions of 15% of the input IκBα complexes used in the binding reaction.

association with phospho-IκBα peptide beads in a phosphorylation-dependent manner in the absence of transfection of β-TRCP (lanes 1–4), but when the levels of β-TRCP were increased by transfection, the quantity of endogenous Skp1 associated with β-TRCP increased substantially (Fig. 3a, lanes 4,6). Analogous results were obtained in a more limited series of binding reactions employing β-catenin-derived peptides (Fig. 3b).

Although it was clear that destruction motif peptides can bind the $SCF^{\beta\text{-}TRCP}$ complex, it was necessary to demonstrate that this complex also recognized the endogenous ubiquitination substrate, the IκK-phosphorylated IκBα/NF-κB complex. To generate this substrate, IκBα/p50/p65 complexes were produced in insect cells and purified to near homogeneity (Fig. 4a). When incubated with ATP and purified IκK-β, the IκBα protein underwent a mobility shift reminiscent of that observed upon phosphorylation in vivo, and this phosphorylated IκBα protein was recognized by phosphospecific antibodies directed at Ser-32 of IκBα (Fig. 4b, lane 2). In addition, microsequencing of IκK-treated IκBα confirmed that both Ser-32 and Ser-36 were phosphorylated (data not shown). To examine whether $SCF^{\beta\text{-}TRCP}$ could recognize this complex, binding reactions were performed using immobilized $SCF^{\beta\text{-}TRCP}$ complexes isolated from 293T cells transiently expressing Myc-tagged β-TRCP or mock transfected cells as a control and either phosphorylated or unphosphorylated IκBα/NF-κB complexes. The $\beta\text{-TRCP}^{Myc}$ immune complexes contain endogenous Skp1 (Fig. 2, lane 3) and Cul1 (data not shown) as determined by immunoblotting. Both IκBα and p50 were found to associate with the $SCF^{\beta\text{-}TRCP}$ complex but not control immune complex in a phosphorylation-dependent manner (Fig. 3c). Similar results were obtained with GST–β-TRCP complexes purified from insect cells (data not shown).

Biochemical association of endogenous IκBα–ubiquitin ligase activity with β-TRCP

Crude cell lysates from the human monocyte cell line THP.1 contain potent IκBα–ubiquitin ligase activity (Fig. 4c). In the context of an IκBα/NF-κB complex, efficient IκBα ubiquitination by these lysates is dependent on phosphorylation by IκK (Fig. 4c). As reported earlier (Yaron et al. 1997), this IκBα–ubiquitin ligase activity is strongly inhibited by phosphorylated IκBα destruction motif peptides (Fig. 4d, lanes 4,5) but not by nonphosphorylatable destruction box peptides (lanes 2,3). Thus, this assay reiterates the requirements for IκBα ubiquitination observed in vivo. Greater than 95% of the IκBα–ubiquitin ligase activity in these extracts can be precipitated with 30%–50% ammonium sulfate (Fig. 4e, lane 1) and can be further purified by chromatography on a phenyl–Sepharose column (Fig. 4e; see Materials and Methods). Peak fractions of IκBα–ubiquitin ligase activity (Fig. 4e, lanes 8,9) elute at 0.5 M ammonium sulfate.

Having partially purified components of the IκBα–ubiquitin ligase, we examined whether β-TRCP and other SCF components were contained in active fractions from the phenyl–Sepharose column. Skp1 has an extended elution profile, but both Skp1 and Cul1 are contained in the active fractions 7 and 8 (Fig. 4e). Skp1 and Cul1 can interact with multiple F-box proteins, and

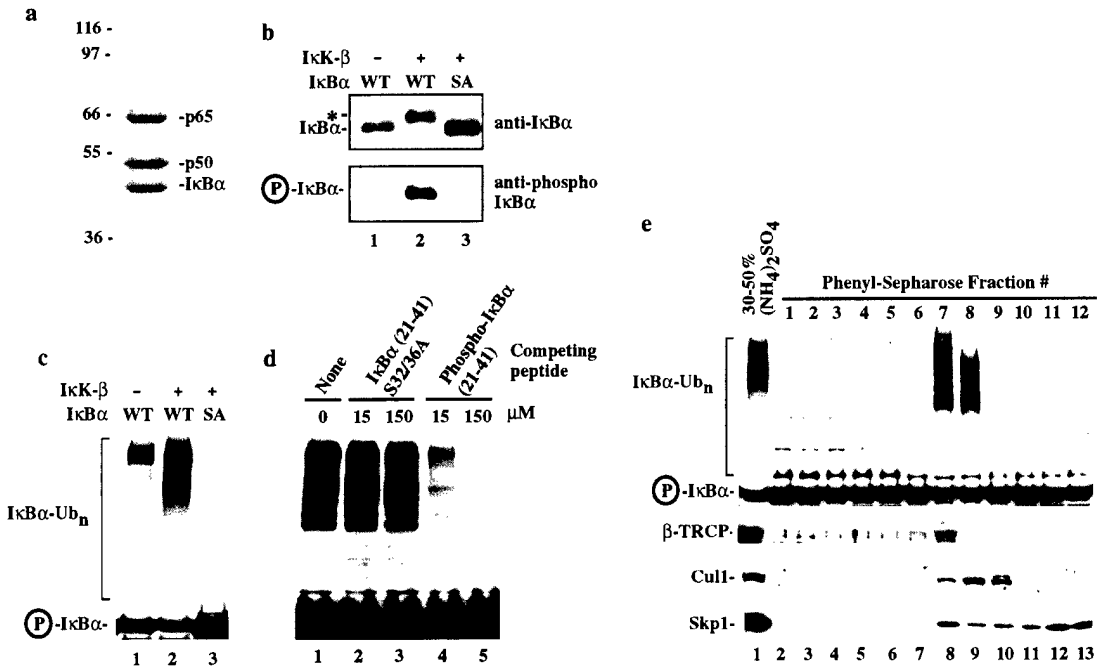

Figure 4. IκBα–ubiquitin ligase activity from human cells cofractionates with β-TRCP. (*a*) IκBα/p50/p65 complexes were purified to near homogeneity from insect cells as described in Materials and Methods. Proteins were separated by SDS-PAGE and stained with Coomassie blue. (*b*) Phosphorylation of the IκBα/p50/p65 complex by IκK-β. The IκBα complex (lanes *1,2*) or a nonphosphorylatable IκBα mutant (S32/36A) complex (lane *3*) was incubated in the presence of ATP and IκK-β as indicated in Materials and Methods. Products were analyzed by immunoblotting with anti-IκBα antibodies to detect a mobility shift accompanying phosphorylation that is absent in the nonphosphorylatable mutant (*top*) or with antibodies that specifically detect the Ser-32-phosphorylated form of IκBα (*bottom*). (*c*) Ubiquitination of IκBα complexes by crude cell lysates was performed as described in Materials and Methods. Phosphorylation leads to a ~10- to 20-fold increase in ubiquitin conjugates relative to the unphosphorylated complex, whereas no activity is observed with the nonphosphorylatable IκBα complexes. (*d*) Inhibition of IκBα ubiquitination by phosphorylated IκBα destruction motif peptides but not by nonphosphorylatable destruction motif peptides (p19). Ubiquitination reactions were performed with crude cell extracts and IκK-β phosphorylated IκBα complexes in the presence or absence of phosphorylated or nonphosphorylatable destruction motif peptides. Specific inhibition of ubiquitination was observed with the phosphorylated peptide. (*e*) Cofractionation of β-TRCP with endogenous IκBα–ubiquitin ligase activity. Crude extracts from THP.1 cells were precipitated with ammonium sulfate. Solubilized proteins containing ubiquitin ligase activity were fractionated using a phenyl–Sepharose column and activity in each fraction determined as described in Materials and Methods. Aliquots of column fractions were assayed for β-TRCP, Cul1, and Skp1 by immunoblotting. Fractions containing β-TRCP, Cul1, and Skp1 (fractions 7,8) contain IκBα–ubiquitin ligase activity.

the identity of the F-box protein in complexes with Cul1 and Skp1 is likely to affect elution properties on phenyl–Sepharose. In contrast with Skp1, β-TRCP levels peak in fraction 7, as determined using affinity-purified carboxy-terminal antibodies, coincident with maximal activity (Fig. 4e, lane 8). β-TRCP was also detected in active fraction 8 (Fig. 4e, lane 9). This fraction contained lower levels of IκBα–ubiquitin ligase activity, as assessed by the extent of conjugation, consistent with the lower levels of β-TRCP. As shown below, under some gel conditions the β-TRCP protein is resolved into a closely spaced doublet of proteins at 58–60 kD. Interestingly, although fraction 6 contains detectable levels of Skp1 and β-TRCP, it lacks detectable Cul1 and IκBα–ubiquitin ligase activity (Fig. 4e, lane 7). Likewise, fraction 9 containing Skp1 and Cul1 but no β-TRCP is also inactive (Fig. 4e, lane 10).

Consistent with a role for Skp1 in the IκBα–ubiquitin ligase, antibodies against Skp1, but not control GST antibodies, deplete IκBα–ubiquitin ligase activity from the active phenyl–Sepharose fractions (Fig. 5a). As expected, Skp1 and Cul1 are largely depleted from these active extracts (lane 2). Importantly, the majority of β-TRCP is also removed by Skp1 antibodies (Fig. 5a, lane 2). β-TRCP migrated as a closely spaced doublet at 58 and 60 kD. The faster migrating form, corresponding to ~80% of the total, was essentially depleted by anti-Skp1 antibodies (lane 2), when compared with control GST-depleted extracts. The source of the heterogeneity in β-TRCP is not known at present, but it is possible that the more slowly migrating form is not associated with Skp1 or is dislodged from Skp1 by the anti-Skp1 antibodies. We also note that Cul1 migrated as a doublet (Fig. 5a). The slower migrating form is likely to correspond to a form of the protein conjugated to NEDD8, a homolog of Rub1 that is known to be covalently linked to Cdc53 in budding yeast (for review, see Hochstrasser 1998).

We also found that phospho-IκBα destruction motif peptides (but not the nonphosphorylatable counterparts) were able to deplete ubiquitin ligase activity from both

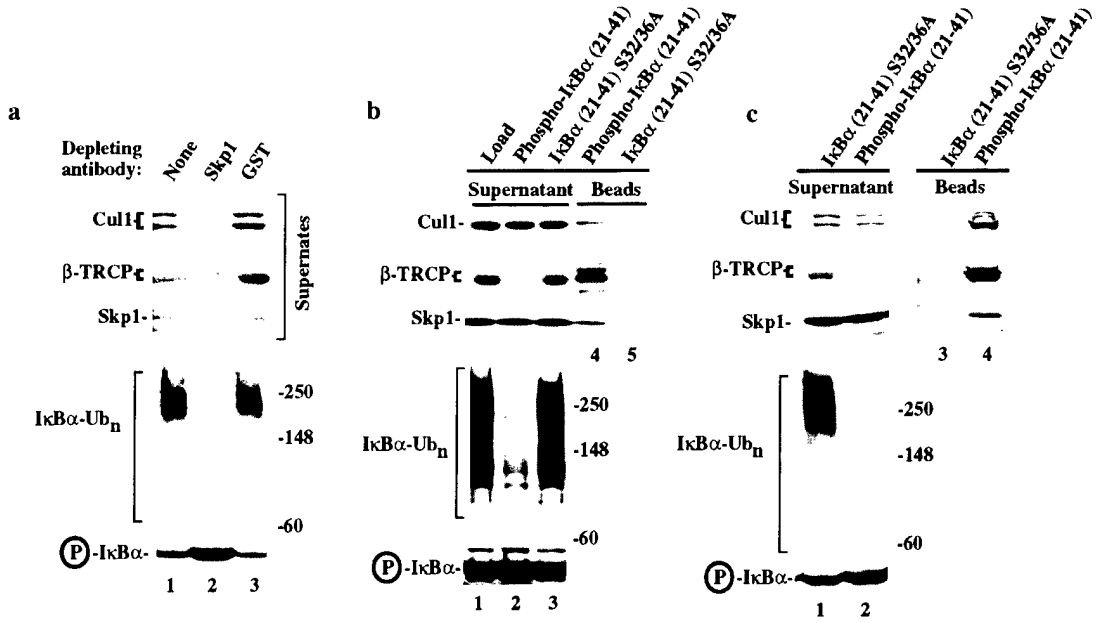

Figure 5. Depletion of IκBα–ubiquitin ligase activity by anti-Skp1 antibodies and destruction motif peptides correlates with removal of β-TRCP. (*a*) Ubiquitin ligase activity from phenyl–Sepharose fractions 7 and 8 was depleted with antibodies against Skp1 or GST and the supernatant assayed for ubiquitination activity toward phosphorylated IκBα (*bottom*). The levels of Skp1, Cul1, and β-TRCP in the supernatants from the depleted fractions were determined by immunoblotting (*top*). (*b*,*c*) Endogenous β-TRCP associates with phosphorylated IκBα destruction motif peptides during depletion of IκBα–ubiquitin ligase activity. Crude cell extracts (*b*) or active fractions from a phenyl–Sepharose column (*c*) were incubated with beads containing either phosphorylated or nonphosphorylatable IκBα peptides and the supernatants assayed for ubiquitin ligase activity (*bottom* panels). The levels of Skp1, Cul1, and β-TRCP in the supernatant and associated with destruction motif peptides were determined by immunoblotting (*top*). β-TRCP is associated with the phosphorylated destruction motif peptides and is substantially depleted from active ubiquitin ligase fractions.

active fractions from the phenyl–Sepharose column (Fig. 5b) and crude cell extracts (Fig. 5c). Skp1, Cul1, and β-TRCP were all associated with the phosphorylated destruction motif beads but not with the nonphosphorylatable destruction motif (Fig. 5, b, lanes 4 and 5, and c, lanes 3 and 4). Although the levels of Skp1 and Cul1 in supernatants were essentially unaffected (Fig. 5, b, lanes 1–3, and c, lanes 1 and 2), the level of β-TRCP in the supernatant from both crude and purified fractions was substantially reduced (~80% for the crude lysate and >90% for the phenyl–Sepharose fraction) (Fig. 5b,c, lanes 1,2). These data are consistent with β-TRCP-associated Skp1 being a small fraction of the total Skp1/Cul1 complexes present in the cell (see Fig. 1b). Currently available antibodies against β-TRCP were unable to immunodeplete β-TRCP from crude lysates or purified fractions, prohibiting a direct analysis of the effects of removal of β-TRCP on IκBα–ubiquitin ligase activity. Nevertheless, the finding that depletion of $SCF^{\beta\text{-}TRCP}$ from either crude lysates or purified fraction correlates with loss of ubiquitin ligase activity strongly implicates this SCF complex as being involved in IκBα ubiquitination.

Stimulation of IκBα ubiquitination by an $SCF^{\beta\text{-}TRCP}$ complex in vitro

The results described thus far are consistent with a role for $SCF^{\beta\text{-}TRCP}$ in IκBα ubiquitination. If β-TRCP functions as a specificity factor for ubiquitination of IκBα through an SCF-dependent pathway, it should be possible to confer IκBα ubiquitination activity by introducing β-TRCP into a system that lacks such an activity. Although budding yeast contains a number of E2 enzymes that could potentially support IκBα ubiquitination, its closest homolog to β-TRCP, Met30, does not associate with the IκBα destruction motif (Fig. 1c). We therefore anticipated that yeast extracts shown previously to support SCF-dependent ubiquitination of Cln2 and Sic1 (Deshaies et al. 1995; Skowyra et al. 1997; Verma et al. 1997) would be inactive toward either phosphorylated or unphosphorylated IκBα and this was the case (Fig. 6c, lanes 4,5). However, when these same reaction mixtures were supplemented with Flag-tagged $SCF^{\beta\text{-}TRCP}$ complexes isolated from 293T cells (Fig. 6a), IκBα ubiquitination was observed (Fig. 6c, lane 7). The activity was dependent upon phosphorylation of IκBα (lane 6) and was absent in reaction mixtures containing anti-Flag immunoprecipitates from mock-transfected cells (lanes 8,9). Moreover, $SCF^{\beta\text{-}TRCP}$ was unable to stimulate ubiquitination when mixed with E1, ATP, and ubiquitin in the absence of yeast extract (lane 10), indicating that a specific E2 activity is not efficiently immunoprecipitated with the $SCF^{\beta\text{-}TRCP}$ complex. As expected, the active $SCF^{\beta\text{-}TRCP}$ complexes associated with phosphorylated IκBα while control immune complex did not (Fig. 6b).

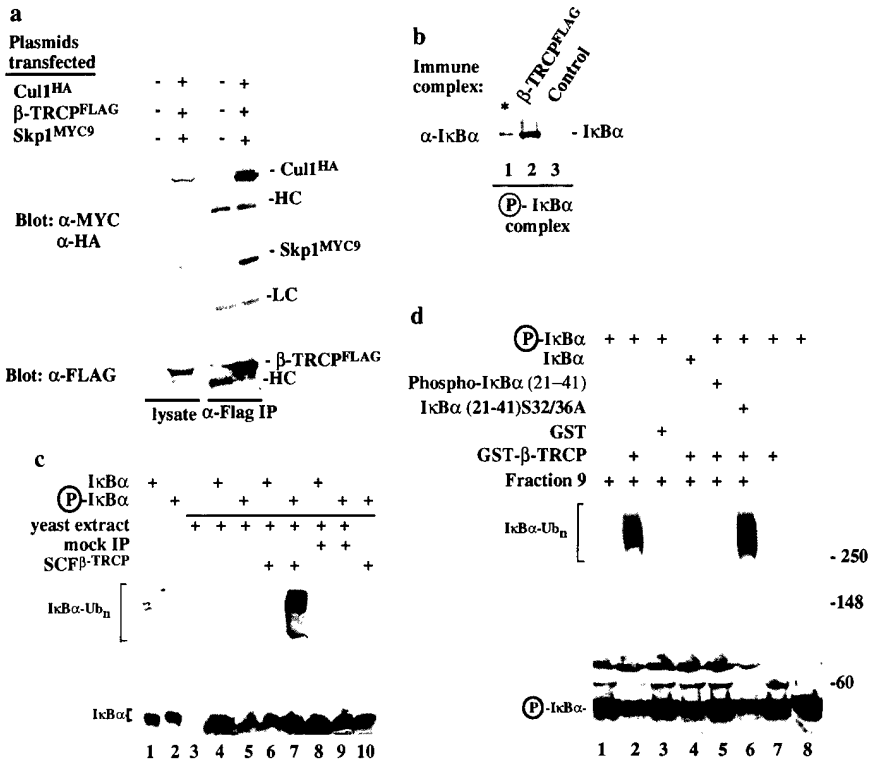

Figure 6. Stimulation of IκBα–ubiquitin ligase activity by SCF$^{\beta\text{-TRCP}}$ in vitro. (*a*) Flag-tagged SCF$^{\beta\text{-TRCP}}$ was prepared after transient transfection in 293T cells by immunoprecipitation along with a Flag immune complex from mock-transfected cells. Immune complexes were analyzed for the presence of Cul1^{HA}, Skp1^{Myc}, and β-TRCP^{Flag} by immunoblotting (lanes *3,4*). Crude lysates used for immunoprecipitation are shown as controls (lanes *1,2*). (*b*) β-TRCP^{Flag} immune complexes associate with phosphorylated IκBα in vitro. Immune complexes (10 μl beads) from (*a*) were incubated with 15 nM phosphorylated IκBα/NF-κB complexes in a total volume of 100 μl. Washed beads were subjected to SDS-PAGE and IκBα determined by immunobloting. The asterisk indicates a sample containing 15% of the input IκBα complex. (*c*) Stimulation of IκBα–ubiquitin ligase activity by SCF$^{\beta\text{-TRCP}}$ in vitro. Yeast extracts (supplemented with E1, ubiquitin, and an ATP-regenerating system) were incubated with unphosphorylated or phosphorylated IκBα/NF-κB complexes (25 nM) in the presence of 10 μl of control immune complexes (lanes *8,9*) or β-TRCP^{Flag} immune complexes (lanes *6,7*). After 90 min, reaction mixtures were separated by SDS-PAGE and IκBα detected by immunoblotting with anti-IκBα antibodies. As controls, untreated IκBα complexes (lanes *1,2*), supplemented yeast lysates (lane *3*), and an SCF$^{\beta\text{-TRCP}}$ immune complex reaction mixture containing all components except the yeast extract (lane *10*) were also included. (*d*) Reconstitution of IκBα ubiquitination activity in mammalian extracts by addition of purified GST–β-TRCP. Reaction mixtures, prepared as described in Materials and Methods, contained E1, ubiquitin, ATP, HQ unbound as a source of E2 activity, and other components as indicated (lanes *1–6*). Control reactions (lanes *7,8*) lacked phenyl–Sepharose fraction 9. After 90 min, products were analyzed by SDS-PAGE and immunoblotting with anti-IκBα antibodies.

In an alternative approach, we tested whether recombinant GST–β-TRCP, purified to near homogeneity from insect cells (data not shown), could support ubiquitination of IκBα in the mammalian ubiquitination system described in Figure 4. As noted above, fraction 9 from the phenyl–Sepharose column contains Cul1 and Skp1 but lacks detectable β-TRCP and IκBα ubiquitination activity (Fig. 6d, lane 1). However, when this fraction was supplemented with GST–β-TRCP, a potent phosphorylation-dependent IκBα–ubiquitin ligase activity was generated (Fig. 6d, lane 2). This activity was not observed when this fraction was supplemented with purified GST protein (Fig. 6d, lane 3). Also, addition of a phosphorylated IκBα destruction motif peptide (but not the unphosphorylated peptide) completely blocked IκBα ubiquitination (Fig. 6d, lanes 5,6). Finally, the activity was not observed when the GST–β-TRCP protein was incubated in the reaction conditions lacking the phenyl–Sepharose fraction, suggesting a requirement for Cul1 and Skp1 (Fig. 6d, lane 7). Taken together, these two assay systems provide compelling evidence that SCF$^{\beta\text{-TRCP}}$ functions as an IκB–ubiquitin ligase.

Discussion

Activation of NF-κB involves an extensive signal transduction pathway that culminates in the destruction of the NF-κB inhibitor IκBα. Although the protein kinase pathways that control the timing of NF-κB activation have been defined, the molecules responsible for the actual ubiquitination events have not been elucidated. In this work, we provide biochemical evidence that the WD-40-containing F-box protein, β-TRCP, functions as a specificity factor in an SCF complex to promote signal-dependent ubiquitination of IκBα (Fig. 7). A role for β-TRCP in controlling IκBα ubiquitination is supported by the following findings. (1) Destruction of IκBα is known to require IκK-dependent phosphorylation of residues (Ser-32 and Ser-36) located in a destruction motif. β-TRCP and its SCF complex associate with this IκBα destruction motif and with the IκBα/NF-κB complex in a manner that is dependent upon phosphorylation of the IκBα destruction motif. A variety of other F-box proteins, including two other WD-40 containing F-box proteins (Met30 and MD6), failed to associate with either phosphorylated or unphosphorylated IκBα destruction motifs, pointing to the specificity of the interaction with β-TRCP. We believe that the interaction between β-TRCP and the IκBα destruction motif is direct, as peptide beads containing this motif precipitate GST–β-TRCP from insect cell lysates in the absence of other abundant proteins (J. Winston, S. Elledge, and J. Harper,

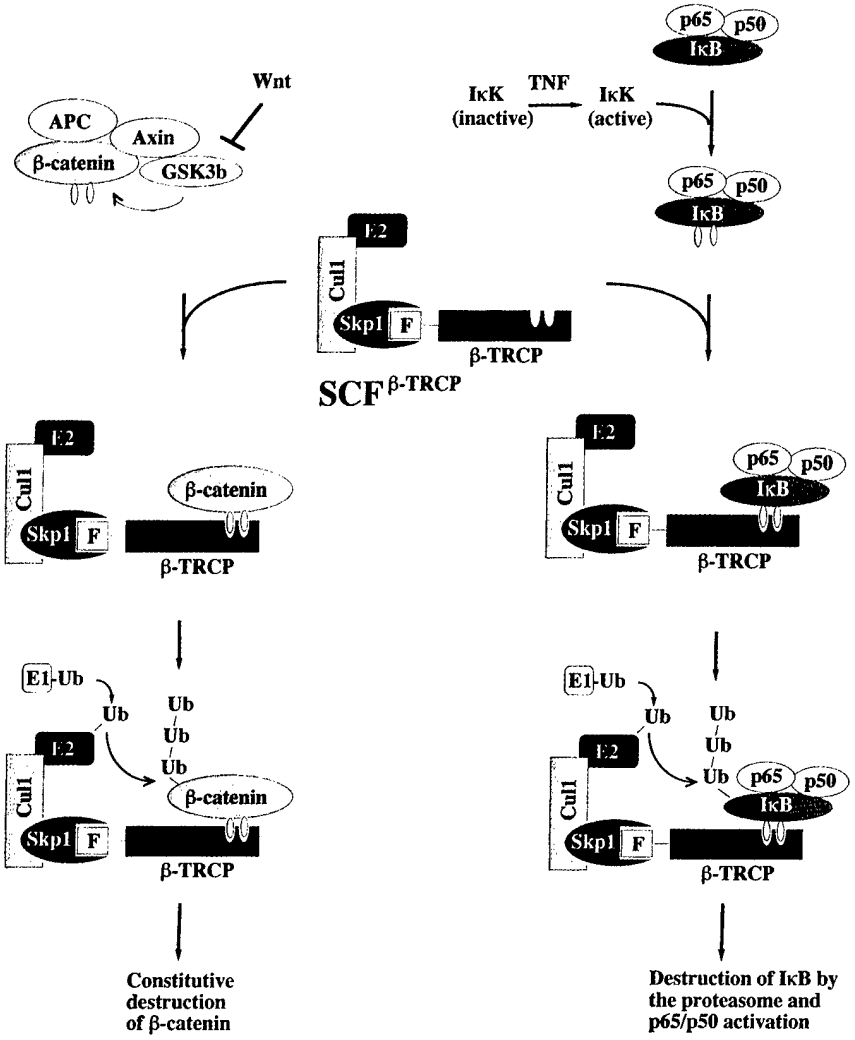

Figure 7. Schematic representation of the proposed pathways controlling ubiquitin-mediated proteolysis of IκBα and β-catenin. β-TRCP, an F-box protein, is a component of an SCF–ubiquitin ligase. In response to appropriate signals (i.e., TNFα), the IκK complex is activated and phosphorylates IκBα in complexes with NF-κB on Ser-32 and Ser-36. This complex is then recognized by β-TRCP in an SCF complex, facilitating ubiquitination by an E1- and E2-dependent mechanism. β-Catenin, in complexes with APC, axin, and GSK3β, is phosphorylated on Ser-33 and Ser-37. This phosphorylated β-catenin can then associate with $SCF^{\beta\text{-TRCP}}$, resulting in ubiquitination. It is not clear at present whether β-catenin alone or the APC/β-catenin complex is the relevant target. Yellow ovals indicate phosphorylation.

unpubl.). (2) β-TRCP forms a complex with two proteins, Skp1 and Cul1, that have been linked previously to phosphorylation-dependent ubiquitination. β-TRCP is localized in the cytoplasm where IκBα ubiquitination is thought to occur. (3) β-TRCP copurifies with IκBα–ubiquitin ligase activity from tissue culture cells, and these active fractions also contain Cul1 and Skp1. (4) Depletion of β-TRCP with either anti-Skp1 antibodies or phosphorylated destruction motif peptides correlates with loss of IκBα–ubiquitin ligase activity. (5) $SCF^{\beta\text{-TRCP}}$ complexes stimulated phosphorylation-dependent IκBα–ubiquitin ligase activity when supplemented with E1, ubiquitin, ATP, and a yeast extract. These yeast extracts lack IκBα–ubiquitin ligase despite the presence of multiple SCF complexes (Bai et al. 1996; Patton et al. 1998a,b), providing further evidence of a role for β-TRCP as a specificity factor for IκBα, but provide E2 activities and possibly other components that support IκBα ubiquitination by the β-TRCP complex. (6) Addition of β-TRCP to fractions containing Cul1 and Skp1 but lacking IκBα–ubiquitination activity leads to robust ubiquitination activity that is phosphorylation dependent and inhibited by a phosphorylated IκBα destruction motif peptide. At present, we have been unable to reconstitute IκBα–ubiquitin ligase activity using $SCF^{\beta\text{-TRCP}}$ complexes isolated from transfected cells and column fractions depleted of β-TRCP by either anti-Skp1 antibodies or phospho-IκBα peptides. This may reflect removal of an essential factor by depletion that is not present in sufficient levels in the transiently expressed SCF complex to support IκBα ubiquitination but are provided in *trans* by yeast extracts or undepleted mammalian extracts. Taken together, these data provide strong evidence that $SCF^{\beta\text{-TRCP}}$ functions in IκBα ubiquitination. After submission of this paper, Yaron et al. (1998) reported that β-TRCP is a component of the IκBα–ubiquitin ligase and demonstrated that mutants lacking the F-box stabilize IκBα and block NF-κB activation in vivo. However, no data linking β-TRCP to an SCF-dependent process was presented, and it was suggested that β-TRCP might function independently of Cul1 and Skp1. Our data provide compelling and complementary biochemical evidence that β-TRCP functions in the context of an SCF pathway, a result that has important mechanistic implications and

further implicates the SCF pathway in phosphorylation-dependent ubiquitination reactions. Currently, the identity of the E2(s) involved in IκBα ubiquitination in vivo is unknown, as is the nature of the heterogeneity observed with β-TRCP. We note, however, that other F-box proteins including Skp2 are modified by phosphorylation (Lisztwan et al. 1998), and such modifications could potentially play regulatory roles. The methods we have employed offer two general approaches for determining whether a particular ubiquitination process involves an SCF complex: (1) Depletion of active fractions with Skp1 antibodies, and (2) the use of substrates as affinity reagents to examine association with cloned F-box proteins. The expanding number of F-box protein sequences available will greatly facilitate the identification of SCF-dependent processes through these types of approaches.

The sequence conservation of the IκBα destruction motif with a region of β-catenin implicated in its turnover, coupled with a genetic requirement for the β-TRCP homolog *slimb* in turnover of the β-catenin homolog Armadillo (Jiang and Struhl 1998), led us to address whether β-TRCP might interact directly with β-catenin. Phosphorylation of serine residues 33 and 37 was sufficient to allow for a peptide spanning this region to associate with β-TRCP and its SCF complex but not other F-box proteins. β-Catenin is a component of the Wingless/Wnt signaling pathway and functions with Tcf/Lef transcription factors to regulate patterning and other developmental decisions (Peifer 1997). Recent work has revealed that expression of a β-TRCP protein lacking the F-box leads to accumulation of β-catenin and ectopic activation of the Wnt pathway in *Xenopus* (Marikawa and Elinson 1998) and β-catenin stabilization in mammalian cells (Latres et al. 1999). This, together with our data linking β-TRCP to direct recognition of the phosphorylated β-catenin destruction motif, strongly implicates SCF$^{\beta\text{-TRCP}}$ as the β-catenin–ubiquitin ligase (Fig. 7). The levels of β-catenin are regulated by the APC (adenomatous polyposis coli) tumor suppressor protein, axin, and the protein kinase GSK3β (Korinek et al. 1997; Morin et al. 1997; Rubinfeld et al. 1997). Formation of an APC/axin/GSK3β/β-catenin complex is thought to be required to allow appropriate phosphorylation of β-catenin by GSK3β (Hart et al. 1998; Ikeda et al. 1998) and in the absence of Wnt signaling, β-catenin levels remain low due to constitutive phosphorylation and ubiquitin-mediated proteolysis. Wnt signaling inactivates GSK3β, leading to increased levels of β-catenin and activation of transcription (Peifer 1997). Mutations in either the APC gene or in β-catenin allow for β-catenin accumulation (Morin et al. 1997; Rubinfeld et al. 1997). Such mutations are found in a large fraction of colon cancers (Morin et al. 1997) and have also been observed in melanoma (Rubinfeld et al. 1997), prostate cancer (Voeller et al. 1998), and experimentally induced cancers (Dashwood et al. 1998). Interestingly, stabilizing mutations in β-catenin are localized to its destruction motif and include mutations in both the phosphoacceptor sites S33 and S37 and other residues in the consensus β-TRCP recognition motif including D32, G34, and I35. Mutations in these residues would be expected to weaken or abolish association of β-catenin with β-TRCP, leading to accumulation of β-catenin.

The role for β-TRCP in β-catenin turnover suggests that it might function as a tumor suppressor. We localized the human β-TRCP gene to 10q24. This region displays genetic abnormalities in a limited number of prostatic, melanocytic, and neural cancers (Parmiter et al. 1988; Lundgren et al. 1992; Rasheed et al. 1992). However, a preliminary analysis of four colon cancers which are wild-type for β-catenin and APC failed to reveal mutations in the β-TRCP protein (Sparks et al. 1998). Given the role for β-TRCP in IκBα ubiquitination, it is conceivable that mutations that inactivate β-TRCP are incompatible with transformation because of loss of survival pathways dependent on NF-κB activation (Beg and Baltimore 1996; Van Antwerp et al. 1996; Wang et al. 1996).

Our results indicate that an SCF$^{\beta\text{-TRCP}}$ complex functions in two critical transcriptional control pathways, in one case by inactivating an inhibitor of transcription and in the other case by inhibiting an activator of transcription (Fig. 7). Genetic data in *Drosophila* suggest that the β-TRCP homolog *slimb* may also regulate the Hedgehog pathway (Jiang and Struhl 1998). In *slimb* mutants, the Ci transcription factor accumulates inappropriately, although the question of whether this regulation is direct remains to be determined. Moreover, other recent studies have revealed that β-TRCP is coopted by the HIV protein Vpu to facilitate destruction of the CD4 protein (Margottin et al. 1998). Interestingly, Vpu contains a β-TRCP recognition motif very similar to that found in IκBα and β-catenin and phosphorylation is required for it to recruit CD4 to β-TRCP and to bind β-TRCP in the two-hybrid system. Further studies are required to determine whether any of the many proteins containing the DSGϕXS motif are also substrates for SCF$^{\beta\text{-TRCP}}$. In addition, we note that the anti-inflammatory effects of aspirin are mediated through inhibition of IκK activity (Grilli et al. 1996; Yin et al. 1998), thereby blocking NF-κB activation. Molecules that selectively block recognition of IκBα by β-TRCP may also constitute an alternative therapeutic target for anti-inflammatory agents.

Materials and methods

Plasmid and baculovirus construction

cDNAs encoding mouse and human homologs of *Xenopus* β-TRCP, human Skp2, and human MD6 were obtained as expressed sequence tags and the sequences determined by automated DNA sequencing. The sequence of human β-TRCP was reported previously. The sequence of mouse β-TRCP was deposited in GenBank (accession no. AF110396). The sources of other novel F-box proteins will be reported elsewhere (J. Winston, D. Koepp, S.J. Elledge, and J.W. Harper, in prep.). Open reading frames were amplified by PCR using Expand high-fidelity polymerase (Boehringer Mannheim) and cloned into the univector derivative pUNI10 as *NdeI–SalI* fragments. Similarly, human Skp1 was cloned into pUNI10 as *NdeI–Bam*HI fragment. In vitro Cre-recombinase-mediated plasmid fusion (Lui et al. 1998) of univector plasmids with various pHOST recipient plas-

mids was then used to create plasmids for expression of proteins as amino-terminal Myc3, Flag, or Ha3 fusion proteins under control of the CMV/T7 promoter, and a GST fusion protein for expression in insect cells. Coding sequences for His_6-tagged IκBα, IκK-β, p50, and p65 were amplified by PCR and baculoviruses generated using the Bac-to-Bac system (Invitrogen). Plasmids for expression of $Cul1^{HA}$ (Lisztwan et al. 1998) and cyclin F (Bai et al. 1996) were from previous studies. GST fusion proteins were purified from insect cells and eluted from glutathione–Sepharose beads as described previously (Skowyra et al. 1997).

Antibodies

Polyclonal antibodies against human Skp1 and Cul1 were generated in rabbits after injection of GST–Skp1 or GST–Cul1(536–815) produced in bacteria. Antisera were depleted of anti-GST reactivity using immobilized bacterial GST protein and then affinity purified using immobilized GST–Skp1 or GST–Cul1(536–815). Antibodies against the carboxyl terminus of β-TRCP were generated in rabbits using the peptide NETRSPSRTYTYISR. Antibodies were affinity purified using this peptide coupled to Affigel-10 (Bio-Rad). Monoclonal antibodies recognizing Myc, Ha, or Flag epitopes were obtained from BabCO or Sigma. Antibodies against the phosphorylated IκBα destruction motif were from New England Biolabs. All other antibodies were from Santa Cruz Biotechnology.

In vitro binding

Twenty-one (p21, residues 21–42) and 19 (p19, residues 23–41) amino acid peptides containing the IκBα destruction motif were synthesized in both the unphosphorylated and doubly phosphorylated forms where serines 32 and 36 are phosphorylated as described previously (Yaron et al. 1997). Analogous unphosphorylated and doubly phosphorylated peptides overlapping a candidate destruction motif in β-catenin were also synthesized. p21 and β-catenin peptides were coupled to Affigel (Bio-Rad) at 2 mg/ml of resin and p19 peptides were coupled to cyanogen bromide activated Sepharose (Pharmacia) at 10 mg/ml resin. Coupling efficiencies were determined to be >90% by analytical reverse-phase HPLC of reaction supernatants. To examine association of Skp1 containing complexes with destruction motifs, binding reactions were performed in a total volume of 120 µl of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.35% NP-40, 1 mM DTT, 0.75 mM EDTA, and 5 µg/ml antipain, leupeptin, and aprotinin using 15 µl of peptide beads and the indicated quantity of cell lysate or column fraction. After incubation at 4°C for ~1 hr, beads were washed three times with 1 ml of binding buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40, 1 mM DTT, 1 mM EDTA, and 5 µg/ml antipain, leupeptin, and aprotinin] prior to SDS-PAGE (13.5%) and immunoblotting using the indicated antibodies. Antibody detection was accomplished using ECL (Amersham). To examine association of F-box proteins with peptide beads, [^{35}S]-methionine-labeled in vitro translation products of various F-box proteins were employed. Comparable quantities of translation products (typically 5–10 µl) were diluted to 120 µl with binding buffer prior to incubation with 15 µl of peptide beads (4°C, 1 hr). Beads were washed three times with binding buffer prior to electrophoresis and autoradiography.

In vivo interactions

To examine assembly of $SCF^{\beta\text{-TRCP}}$ complexes in mammalian cells, 293T cells were transfected with various combinations of plasmids (1 µg each/10-cm dish) expressing Myc, Flag, or Ha-tagged Skp1, β-TRCP (mouse), and Cul1 under control of the CMV promoter using lipofectamine (GIBCO/BRL). The total DNA level was kept constant at 10 µg using empty vector DNA and each transfection contained 100 ng of pCMV–GFP DNA to facilitate determination of transfection efficiency (typically >90%). Forty hours post-transfection, cells were lysed in binding buffer supplemented with 10 mM β-glycerol phosphate, 5 mM NaF, and 1 mM *p*-nitrophenylphosphate (30 min on ice). After centrifugation (14,000 rpm, 20 min), lysates (1 mg/0.5 ml) were subjected to immunoprecipitation using 10 µl of anti-Myc or anti-HA immobilized on agarose beads, or used in peptide bead binding reactions as described above. Washed immune complexes were separated by SDS-PAGE and immunoblotted using the indicated antibodies. In some experiments, immune complexes from transfected cells were used for ubiquitination reactions (see below) or for binding to IκBα/NF-κB complexes. For binding experiments, immobilized SCF complexes from one 10-cm dish of transfected 293T cells (10 µl of beads) were incubated with 15 nM IκBα/NF-κB complexes in a total volume of 100 µl of binding buffer for 60 min. Beads were washed three times with 600 µl of binding buffer prior to immunoblotting for p50 and IκBα.

In vitro ubiquitination

To generate IκBα/NF-κB complexes as a substrate for ubiquitination, insect cells were coinfected with viruses expressing His_6–IκBα, p50, and p65 and complexes purified on nickel–NTA resin. Proteins were eluted with imidazole and the heterotrimeric complex size-selected by gel filtration. To generate phosphorylated IκBα, the IκBα/p50/p65 complex (3 µM) was incubated with IκK-β (500 nM) purified from insect cells and 300 µM ATP, 10 mM $MgCl_2$, 1 mM DTT, 50 mM Tris (pH 7.5) for 2 hr at 25°C. IκBα–ubiquitin ligase activity in THP.1 cell extracts and column fractions was assayed as described previously (Chen et al. 1995) using 25 nM phosphorylated or unphosphorylated IκBα/NF-κB complexes, HQ unbound (33 µg), and 200 nM E1. Proteins were separated by 4%–20% Novex SDS-PAGE, transferred to nitrocellulose, and IκBα revealed with anti-IκBα antibodies. THP.1 cell extracts were prepared by Dounce homogenization (10 strokes) in eight volumes (wt/vol) of buffer containing 50 mM Tris, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 1× Boehringer Mannheim protease inhibitor cocktail (EDTA-free) and centrifuged at 25,000g for 25 min. The filtered supernatant was fractionated on a POROS HQ column equilibrated in dialysis buffer lacking glycerol to obtain unbound fraction (HQ unbound) or precipitated with ammonium sulfate. The 30%–50% ammonium sulfate cut equivalent to 1 gram of cell paste was solubilized in 3 ml of dialysis buffer (25 mM Tris at pH 7.5, 10% glycerol, 1 mM DTT, 1 mM Benzamidine, 0.4 mM PMSF). The solubilized pellet (2 ml) was adjusted to 1.2 M ammonium sulfate, loaded to phenyl–Sepharose column (Pharmacia 5/5) equilibrated in dialysis buffer containing 1.2 M ammonium sulfate but without glycerol, and bound proteins eluted with a 20 column volume gradient 1.2–0 M ammonium sulfate, collecting two-column volume fractions followed by dialysis. To deplete IκBα–ubiquitin ligase activity from crude extracts (5 mg/ml) or phenyl–Sepharose fractions (0.7 mg/ml), p19 or pp19 beads (5 µl) were incubated with 30 µl of sample for 2 hr. Unbound material was removed for ubiquitination assays or immunoblotting and proteins associated with washed beads were subjected to SDS-PAGE and immunoblotting. Skp1 was immunodepleted by precoupling 30 µl of protein G beads (Pierce) with 1 ml of anti-Skp1

or anti-GST mouse monoclonal IgG_1 antibodies (Transduction Laboratories) at 45 μg/ml for 2 hr. Washed beads were incubated with phenyl–Sepharose pools for 3 hr as indicated above.

To assay IκBα ubiquitination activity in yeast extracts, 50 μg of crude yeast extract (Deshaies et al. 1995) was supplemented with 100 ng of human E1, 200 μg of ubiquitin, 2 mM ATP (together with an ATP regenerating system), 10 μM LLNL, and 25 nM of IκBα/NF-κB complexes as substrate in a total volume of 10 μl (50 mM Tris-HCl at pH 7.5, 5 mM $MgCl_2$, 0.6 mM DTT). Reaction mixtures were incubated with SCF immune complexes or control immune complexes from transfected 293T cells (10 μl of beads per assay) for 90 min at 30°C and products analyzed by SDS-PAGE and immunoblotting with antibodies against IκBα. We estimate that the reaction mixture contained 50–100 ng of SCF complexes. To assay IκBα ubiquitination activity in reconstituted mammalian extracts, GST–β-TRCP or GST proteins purified from insect cells (200 ng) were added to 100 μg of phenyl–Sepharose fraction 9 followed by HQ unbound (33 μg), E1 (200 ng), ubiquitin (5 μM), and ATP (2 mM) in a final volume of 15 μl (90 min). In some cases, IκBα peptides were added at 50 μg/ml.

Chromosomal localization and in situ hybridization

Genomic clones (Bacmids 171I9 and 350H4) that hybridize to the human β-TRCP cDNA were obtained from Research Genetics. The chromosomal localization of the human β-TRCP gene was determined by fluorescence in situ hybridization by the Baylor College of Medicine FISH facility. RNA expression analysis by in situ hybridization was performed as described (Parker et al. 1995) using antisense mouse β-TRCP sequences as a probe and sense sequences as a negative control (data not shown).

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A family of mammalian F-box proteins

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Ubiquitin-mediated destruction of regulatory proteins is a frequent means of controlling progression through signaling pathways [1]. F-box proteins [2] are components of modular E3 ubiquitin protein ligases called SCFs, which function in phosphorylation-dependent ubiquitination ([3–5], reviewed in [6,7]). F-box proteins contain a carboxy-terminal domain that interacts with substrates and a 42–48 amino-acid F-box motif which binds to the protein Skp1 [2–4]. Skp1 binding links the F-box protein with a core ubiquitin ligase composed of the proteins Cdc53/Cul1, Rbx1 (also called Hrt1 and Roc1) and the E2 ubiquitin-conjugating enzyme Cdc34 [8–11]. The genomes of the budding yeast *Saccharomyces cerevisiae* and the nematode worm *Caenorhabditis elegans* contain, respectively, 16 and more than 60 F-box proteins [2,7]; in *S. cerevisiae*, the F-box proteins Cdc4, Grr1 and Met30 target cyclin-dependent kinase inhibitors, G1 cyclins and transcriptional regulators for ubiquitination ([3–5,8,10], reviewed in [6,7]). Only four mammalian F-box proteins (Cyclin F, Skp1, β-TRCP and NFB42) have been identified so far [2,12]. Here, we report the identification of a family of 33 novel mammalian F-box proteins. The large number of these proteins in mammals suggests that the SCF system controls a correspondingly large number of regulatory pathways in vertebrates. Four of these proteins contain a novel conserved motif, the F-box-associated (FBA) domain, which may represent a new protein–protein interaction motif. The identification of these genes will help uncover pathways controlled by ubiquitin-mediated proteolysis in mammals.

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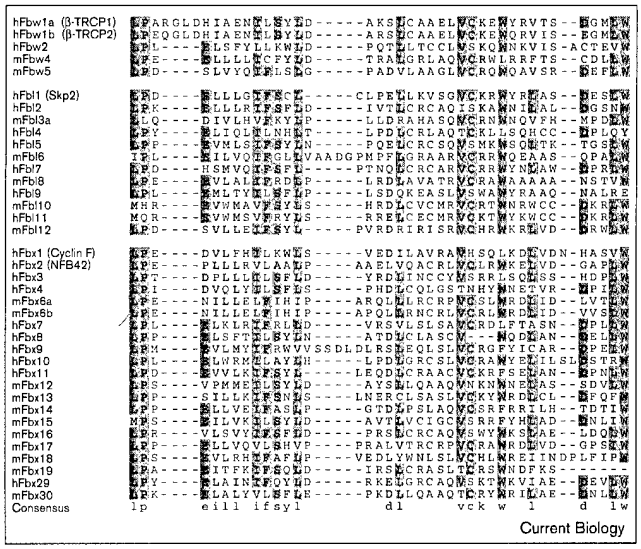
Results and discussion

Using yeast two-hybrid screens to isolate proteins that bind Skp1 and by searching the expressed sequence tag (EST) database, we identified 33 cDNAs encoding novel mammalian F-box proteins (Figure 1). In 24 cases, both human and rodent homologs were identified, and they were typically > 90% identical (data not shown). Cenciarelli *et al.* [13] and Regan-Reimann *et al.* [14] have independently identified a partially overlapping set of human and *Xenopus* F-box proteins. A composite alignment of 50 mammalian F-box motifs is included in the Supplementary material.

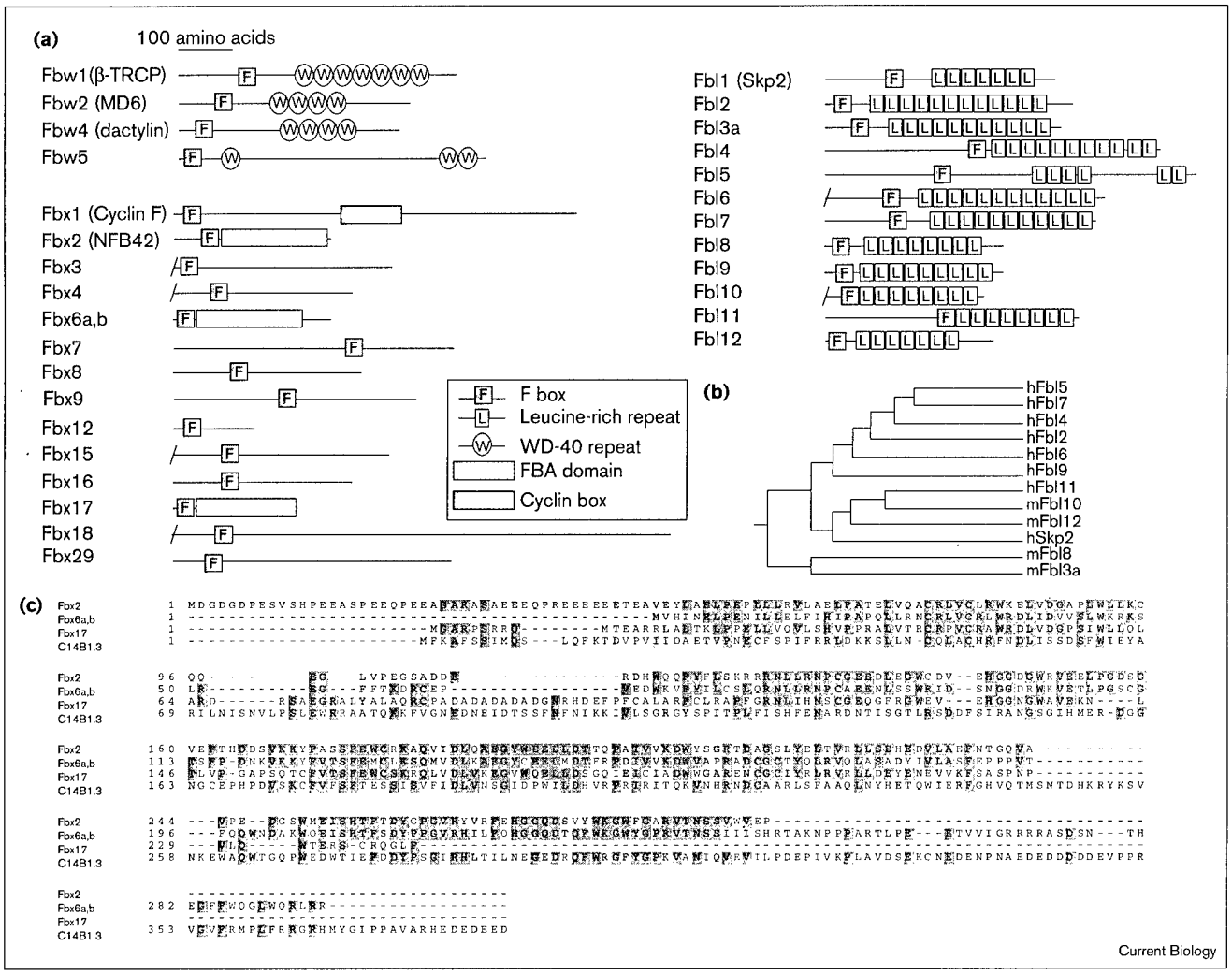
Sequence analysis revealed three subfamilies of F-box proteins: Fbws contain WD-40 repeats, Fbls contain leucine-rich repeats, and Fbxs lack known protein-interaction domains (Figures 1,2a); β-TRCP, Skp2 and Cyclin F, respectively, are the first members of the three subfamilies, and the two close homologs of β-TRCP will be referred to as Fbw1a and Fbw1b. In agreement with Cenciarelli *et al.* [13], the other members have been numbered in order of discovery.

Figure 1



A family of mammalian F-box proteins. Sequences of novel F-box motifs and comparison with F-box motifs in Cyclin F (Fbx1), Skp2 (Fbl1), β-TRCP1 (Fbw1a), β-TRCP2 (Fbw1b) and NFB42 (Fbx2). The alignment was performed using Clustal W 1.7. Dark gray, identical residues; light gray, similar residues; h, human; m, mouse. Fbx3 was identified from a human breast cDNA library using Skp1 as bait in a yeast two-hybrid screen. Other novel F-box sequences were obtained by performing iterative BLAST searches of the GenBank database using sequences of newly-identified F-box motifs. GenBank accession numbers are provided in the Supplementary material or can be obtained by querying GenBank at http://www.ncbi.nlm.nih.gov/genbank/query_form.html.

Figure 2



(a) Domain structures of F-box proteins. **(b)** Phylogenetic analysis of Fbl proteins, performed using the DNAStar software package. **(c)** Alignment of those members of the Fbx subfamily that have FBA domains: Fbx2, Fbx6a, Fbx17 and the hypothetical *C. elegans* protein C14B1.3. Dark gray, identical residues; light gray, similar residues

The Fbls comprise the largest subfamily of F-box proteins, with 12 members (Figure 2a) that, on the basis of phylogenetic comparisons, cluster into three groups (Figure 2b). Fbl2, Fbl5 and Fbl7 display extensive internal homology within the leucine-rich repeats and are most closely related to Grr1, which is involved in G1 cyclin ubiquitination in budding yeast [3,6,8,10], and the hypothetical F-box protein C02F5.7 in *C. elegans*. Other Fbl proteins display little internal similarity in their leucine-rich repeats or with one another outside of the LXL motifs (in the single-letter amino-acid code, where X is any amino acid) that define this domain. Fbw2 and Fbw4 contain multiple WD-40 repeats but display no significant sequence similarities with each other or with other proteins in the database outside these motifs (Figure 2a). Fbw5 contains three WD-40 repeats, the first one separated by a large spacer region from the other two. Recently, mutations in Fbw4 were shown to be responsible for dactylaplasia in mice, a condition that resembles split-hand split-foot malformation-3 in humans [15].

The Fbx subfamily are a diverse set of proteins, lacking defined protein–protein interaction domains carboxy-terminal to the F box, with the exception of the cyclin box found in Cyclin F (Figure 2a). Nevertheless, four members displayed similarity to each other and were characterized by a carboxy-terminal motif, which we call the F-box-associated (FBA) domain, possibly representing a new protein–protein interaction motif (Figure 2a,c). The founding member of this group is Fbx2 (NFB42), recently

reported to be abundant in neurons [12]. We found three additional mammalian homologs of Fbx2: the two closely related proteins Fbx6a and Fbx6b and the more distantly related Fbx17 (Figure 2a,c). *C. elegans* has two hypothetical F-box proteins (C14B1.3 and T01E8.4), which have FBA-like domains (Figure 2c and data not shown).

The F-box motif is functionally defined as a motif that can interact with Skp1 [2]. We found that 12 randomly selected F-box proteins from all three subfamilies (Fbw2, Fbw5, Fbl2, Fbl4, Fbl5, Fbl8, Fbx3, Fbx7, Fbx8, Fbx12, Fbx15, Fbx16) associate with a fusion protein between glutathione-S-transferase and Skp1 (GST–Skp1) *in vitro* (see Supplementary material) and with Skp1 in transfected 293 cells (data not shown). These 12 proteins are widely expressed during mouse embryogenesis and in adult tissues, and preliminary studies indicate that the majority are located in the cytoplasm when expressed transiently in 293 cells (see Supplementary material).

In summary, mammalian F-box proteins represent an expanding family of proteins and additional members are likely to emerge with time. Currently, F-box proteins are the largest class of E3 ubiquitin ligase receptors and, as many F-box proteins recognize multiple substrates, the SCF system may be able to ubiquitinate hundreds of proteins. Some F-box proteins may themselves be targeted for ubiquitination through association with the Skp1–Cul1 complex [2,16]. Recent data suggest that other homologs of cullin proteins may be involved in ubiquitin ligase systems [9,11] that are also combinatorial in nature. For example, the Cul2–Elongin BC complex interacts with a family of SOCS-box proteins (typified by the Suppressor of cytokine signalling-1 protein), of which there are currently 20 members [17,18]. The identification of mammalian F-box proteins will facilitate both the elucidation of pathways that are controlled by the SCF system and the identification of particular F-box proteins that recognize known ubiquitination targets. For example, surveys of a collection of F-box proteins for interaction with ubiquitination substrates has led to the identification of β-TRCP (Fbw1a) as the mediator of IκBα and β-catenin ubiquitination [19], and Skp2 (Fbl1) as the mediator of p27 ubiquitination [20]. Identification of targets of the family of mammalian F-box proteins reported here will be a challenge for the future.

Supplementary material

Supplementary material including figures showing alignment of leucine-rich repeats, association of F-box proteins with Skp1, subcellular localization and expression patterns; GenBank accession numbers; primer sequences; and a compilation of F-box sequences from this paper and [13] is available at http://current-biology.com/supmat/supmatin.htm.

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